In vitro biofilm formation by *Streptococcus pneumoniae* as a predictor of post-vaccination emerging serotypes colonizing the human nasopharynx

Mirian Domenech,[1,2*] Lidia Araújo,[1] Ernesto García,[1,2] and Miriam Moscoso[1,2]

[1] *Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, Spain.*

[2] *CIBER de Enfermedades Respiratorias (CIBERES).*

Running title: Pneumococcal serotypes and biofilm formation

For correspondence. *E-mail mirid@cib.csic.es; Tel. (+34) 91 8373112; Fax (+34) 91 5360432.

Summary

The increasing use of the 7-valent pneumococcal conjugate vaccine has been accompanied by the rise of nonvaccine serotypes colonizing the human nasopharynx. The vast majority of infections are caused by microorganisms that grow in biofilms. It has recently been shown that the formation of *Streptococcus pneumoniae* biofilms *in vivo* and *in vitro* is hindered by the presence of capsular polysaccharide. The biofilm-forming capacity of pneumococcal clinical isolates with different types of capsular polysaccharide and various isogenic transformants was examined. Strains of serotypes 19A and 19F, but not 19B and 19C, formed ≥80% of the quantity of biofilm associated with a non-encapsulated control strain. Strains of serogroup 6 also showed significant biofilm-forming capacity. The capsules of serotypes 19A and 19F and serogroup 6 contain the disaccharides α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→ and α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→. Serotype 18A and serotypes 18B/18C have very similar capsular disaccharides: α-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→ and α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→, respectively. However, the strains of serogroup 18 showed impaired biofilm formation. These results indicate that the chemical composition/structure of the capsular polysaccharide is crucial to the biofilm-forming capacity of pneumococcal serotypes. Testing of the *in vitro* biofilm-forming ability of isogenic transformants expressing different capsular polysaccharides may help predict the emergence of colonizing, nonvaccine serotypes.

Introduction

Streptococcus pneumoniae (pneumococcus) is responsible for episodes of acute otitis media (AOM), pneumonia, bacteraemia and meningitis, mainly in children, the elderly and the immunocompromised, and may be directly responsible for as many child deaths as HIV/AIDS, malaria and tuberculosis combined (O'Brien *et al*., 2009). The 23-valent pneumococcal polysaccharide vaccine (PPSV23), introduced in 1983, is recommended for all adults aged 65 years and older or adults younger than age 65 years with chronic lung disease, chronic cardiovascular diseases, cirrhosis, diabetes mellitus, functional or anatomic asplenia, etc., as well as for children over 2 years of age who were at a substantially increased risk of developing pneumococcal infection (Centers for Disease Control and Prevention (CDC), 2013). A 7-valent conjugate vaccine (PCV7) was recommended for universal use in children aged 23 months and younger in the United States in 2000 (American Academy of Pediatrics – Committee on Infectious Diseases, 2000). In the following years its use began in Europe (2001) and other parts of the world. PCV7 contains conjugate capsular polysaccharide directed at 7 (4, 6B, 9V, 14, 18C, 19F and 23F). Since 2000, the widespread use of PCV7 for children has reduced the incidence of pneumococcal disease in all age groups (Whitney et al., 2003). A few years after its introduction, the rates of AOM and invasive pneumococcal disease (IPD) caused by PCV7 serotypes in children under 5 decreased dramatically (Nuorti *et al*., 2010).

It is now clear, however, that the pneumococcal population has changed since the widespread introduction of PCV7. Non-PCV7 serotype isolates have increased among asymptomatic carriers in a process known as “serotype replacement” and have become increasingly responsible for cases of IPD (Weinberger *et al*., 2011). Although some PCV7 serotypes appear to offer cross-protection against certain non-PCV7 serotypes, e.g., 6B offers protection against 6A, this has not been clearly demonstrated for 19F and 19A (Hausdorff *et al*., 2010). Indeed, the incidence of infections caused by multiresistant 19A pneumococci has increased since PCV7 vaccination became

common (Liñares *et al*., 2010; Richter *et al*., 2013). In the pre-PCV7 era, 19F and 19A were among the most common serotypes involved in AOM worldwide (Rodgers *et al*., 2009). Nowadays, multiresistant *S. pneumoniae* 19A isolates remain the most commonly encountered in many countries, while the number of 19F infections has greatly diminished (Hanage *et al*., 2011; Spijkerman *et al*., 2011). Evidence that serotype 19A is well capable of colonizing the nasopharynx and causing AOM has recently been reported using a chinchilla otitis media model (Laufer *et al*., 2010). The explanation for the notable colonizing capacity of serotypes 19F and 19A remains unclear, although a direct relationship between the success of a serotype during nasopharyngeal carriage and its capsular polysaccharide (CPS) biochemistry has been suggested (Weinberger *et al*., 2009). It has also been reported that CPS biosynthesis interferes with pneumococcal growth in a nutritionally limited environment (as the nasopharynx is thought to be), probably by competition for energy with the organism's central metabolism. This suggests that the serotype-specific nasopharyngeal carriage prevalence *in vivo* is predicted by the growth phenotype (Hathaway *et al*., 2012).

The vast majority of infections are now thought to be caused by microorganisms growing as part of a biofilm (Wolcott and Ehrlich, 2008). A biofilm is a thin layer of microorganisms embedded in an extracellular matrix that adheres to the surface of an organic or inorganic structure. Recent reports have documented the *in vivo* formation of *S. pneumoniae* biofilms on adenoid and mucosal epithelial tissues in children with recurrent or chronic ear infections (for a recent review, see (Domenech et al., 2012). A number of laboratories have also shown that both the *in vivo* and *in vitro* formation of pneumococcal biofilms is hindered by the presence of CPS, *i.e.*, the presence of a capsule inhibits pneumococcal biofilm development (Domenech *et al*., 2012). Indeed, encapsulated clinical pneumococcal isolates and isogenic transformants form significantly less biofilm than non-encapsulated strains (Moscoso *et al*., 2006), and an inverse relationship is reported between the ability of non-encapsulated variants to form biofilms and the amount of CPS they produce (Domenech *et al*., 2009). These results agree with the proposal that pneumococci regulate CPS expression in their transition

from nasopharyngeal carriage associated with biofilm development to IPD (Hammerschmidt *et al*., 2005).

In contrast to that previously thought, this paper reports that clinical isolates of serotypes 19F and 19A (but not 19B or 19C), as well as isogenic transformants expressing the CPS of 19F or 19A, are good biofilm producers. This may explain the prevalence of these serotypes in the human nasopharynx. Studies with other serogroups suggest that the presence of a specific disaccharide in the CPS may be important for promoting biofilm formation.

Results

Biofilm-forming capacity of serogroup 19 strains

S. pneumoniae serogroup 19 is composed of four cross-reacting serotypes: 19F, 19A, 19B and 19C (the latter two seldom cause disease). The repeating units of the 19F and 19A CPSs are very similar to those of 19B and 19C, although the last two have some extra sugars (Fig. 1), and the immunochemical similarities of all four inferred from cross-reactivity with factor typing sera (Beynon *et al*., 1992). As expected, the complexity of their chemical structures is reflected in that of the corresponding capsular gene clusters (Beynon *et al*., 1992). The immunological cross-reactions revealed by the capsular reaction showed that serotypes 7B, 7C, 19B, 19C, 24F, 24B and 40 have a common antigenic determinant known as 7h (Henrichsen, 1995). Comparisons with the primary structures of serotypes 19B and 19C agree with a previous proposal (Beynon *et al*., 1992) that the 7h antigen corresponds to the disaccharide element β-D-Rib*f*-(1→4)-α-L-Rha*p*-(1→ (Fig. 1).

Figure 2 shows the biofilm-forming capacity of different serogroup 19 isolates. With the exception of strains SSISP19F/1, G54 and 1061, which were intermediate biofilm formers, most clinical strains of serotypes 19F and 19A formed ≥80% of the quantity of biofilm produced by the nonencapsulated control strain M11. As expected, strain P015 (a serotype 23F transformant of M11 included as an additional control) formed very

little biofilm. In sharp contrast with serotype 19F and 19A strains, those belonging to the rare serotypes 19B and 19C behaved like most encapsulated pneumococci, producing only a small number of biofilm-associated sessile cells.

Given the reported inhibitory effect of CPS on biofilm formation, the biofilm-forming behavior of the 19F and 19A clinical isolates was unexpected. However, *S. pneumoniae* isolates show wide genetic variability beyond that of CPS formation (Croucher *et al*., 2011), and the genetic background of an isolate may strongly influence its capacity to form a biofilm (Domenech *et al*., 2012). To reduce the influence of genetic variability, isogenic capsular transformants producing serogroup 19 capsules were constructed using the nonencapsulated M11 strain as a recipient (Table 1). The biofilm-forming capacity of the corresponding transformants almost paralleled those of the corresponding parental clinical isolates (Fig. 2). Notably, strain P192 formed much more biofilm than its parent G54, confirming the importance of genetic background in biofilm development.

The distinctive biofilm-forming behavior of the 19F isolates was further examined by construction of strain P194, a binary encapsulated transformant of strain P192 that simultaneously expressed the CPS of serotypes 19F and 37. Pneumococci expressing serotype 37 CPS are virtually unable to form biofilms (Moscoso *et al*., 2006), so it was expected that the transformant would show a drastic reduction in biofilm-forming capacity compared to the encapsulated P192 parental strain (Fig. 2).

Together, the above results indicate that, in addition to genetic background, the chemical composition/structure of the CPS is crucial to biofilm-forming capacity. A close comparison of the primary structures of the repeating units of the CPS of serotypes 19F and 19A on one hand, and 19B and 19C on the other, suggest that the disaccharides α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→ and α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→ (present in serotype 19F and 19A CPSs respectively, but not in 19B and 19C capsules) may be important for promoting biofilm formation.

Biofilm-forming capacity of other pneumococcal CPSs with disaccharides similar to α-

*D-Glc*p-*(1→3)-α-L-Rha*p

The disaccharide α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→ is present in the CPS of the four known serotypes of serogroup 6 (Fig. 3A). Moreover, the CPSs of all four are characterized by the presence of a rhamnose–ribitol linkage. However, the CPSs of 6B and 6D have a 1→4 rhamnose–ribitol linkage, whereas those of 6A and 6C have a 1→3 rhamnose–ribitol linkage; 6A and 6B CPSs also contain galactose, while those of 6C and 6D do not (Bratcher *et al*., 2010).

We previously reported (Moscoso *et al*., 2006) that a serotype 6B clinical isolate and an M11 transformant expressing the 6B capsule showed some 70% of the biofilm-forming capacity of the unencapsulated M11 strain. The analysis of other members of this serogroup, namely serotypes 6A and 6C (no serotype 6D isolate was available) showed that both clinical isolates and isogenic transformants expressing these capsules formed ≥40% of the quantity of biofilm formed by the nonencapsulated control strain (Fig. 3B). These results agree with the hypothesis that the presence of α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→ in the CPS is strongly related to biofilm-forming capacity.

Dob1 has long been recognised as a hybridoma-secreted human immunoglobulin G2 antibody that binds to and opsonizes pneumococci of serogroup 6 (Sun *et al*., 1999). It can bind to synthetic capsular carbohydrates α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol and α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol, but not to serotype 2 and 19F CPSs, which contain β-D-Glc*p*-(1→3)-α-L-Rha*p* and α-D-Glc*p*-(1→2)-α-L-Rha*p* determinants respectively (Park *et al*., 2009). The epitope α-D-Glc*p*-(1→3)-α-L-Rha*p* is present in the CPS of serotypes 6A, 6B, 6C, 6D and 19A, but not in that of 19F. Purified Dob1 was added to strain P181 (a serotype 19A transformant) in a standard biofilm-forming assay. When Dob1 was used in amounts that did not cause growth inhibition, no changes in biofilm formation were seen (data not shown).

The biofilm-forming capacity of serotypes 18A, 18B and 18C was also investigated. Serotype 18A CPS contains the disaccharide α-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→, whereas serotypes 18B and 18C CPSs have α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→, very

similar to those present in serogroups 6 and 19 (Fig. 4A). In addition, serotype 18C has a glycerol-phosphate substituent required for the antigenicity of the 18C CPS to be conserved (Chang *et al*., 2012). Among all the members investigated of this serogroup, only one clinical isolate of serotype 18C was able to form a substantial amount of biofilm, but the corresponding M11 transformant (strain P202) showed no significant biofilm-forming capacity (Fig. 4B).

Discussion

The antiphagocytic properties of *S. pneumoniae* CPS are essential for it to show systemic virulence (Hyams *et al*., 2010). At least 95 different pneumococcal serotypes are known, each with a biochemically distinct CPS. The most recent descriptions include serotypes 6E (Ko et al., 2013), 6F and 6G (Oliver et al., 2013a). It is likely that additional serotypes remain to be described (note that serological heterogeneity among serotype 20 isolates has recently been reported (Burton and Nahm, 2012)). There is also growing evidence that pneumococci can change their CPS structure and, consequently, their immunological properties through minimal genetic modifications (Oliver et al., 2013b; Oliver et al., 2013a). The distribution of serotypes worldwide is also continuously evolving since it depends on the year, country, age of the individual and site of isolation, and use of antibiotics. The widespread use of PCV7 for the immunization of children has strongly modified the prevalence of *S. pneumoniae* serotypes; those covered by PCV7 have declined while nonvaccine serotypes have become more common. In particular, the noticeable proliferation of multiresistant 19A pneumococci has been associated with PCV7 use (Hulten *et al*., 2013), although increases had been reported before the introduction of the vaccine (Dagan *et al*., 2009), perhaps as a result of antibiotic pressure (Hulten *et al*., 2013).

The present results show that clinical isolates as well as isogenic pneumococcal transformants of serotypes 19F or 19A (but not those of serotypes 19B or 19C) are capable of forming substantial amounts of biofilm *in vitro*. Comparisons of the primary

structure of the repeating units of the corresponding CPSs suggest that the presence of disaccharides α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→ (serotype 19F) and α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→ (serotype 19A) may stimulate biofilm formation in some way. In agreement with this idea, *S. pneumoniae* serogroup 6 strains, which also contain the latter disaccharide in their CPSs, were seen to be noticeable biofilm formers. The serogroup 18 isolates, however, showed no such behaviour. The CPS of serogroup 18 pneumococci contains β-L-Rha*p* instead of the α-L-Rhap seen in the CPSs of serotypes 19F/19A and serogroup 6.

Other factors may also affect biofilm formation, e.g., the primary structure of an oligosaccharide and its three-dimensional folding. However, the binding of the Dob1 antibody, which recognizes the critical epitope α-D-Glc*p*-(1→3)-α-L-Rha*p* (Park *et al*., 2009), did not modify the biofilm-forming capacity of a 19A pneumococcal strain, at least under the experimental conditions used here.

The two serotypes that show the strongest colonization capacity, 19A and 6C (Laufer *et al*., 2010), are not covered by the PCV7 vaccine. Following the introduction of PCV7, serotype 19A has emerged as one of the most commonly encountered clinical serotypes (Hulten *et al*., 2013). It has been suggested that any effect of PCV7 on 19A would be limited to disease prevention once colonization has occurred, rather than any prevention of colonization itself (Hausdorff *et al*., 2010). The discovery of the strong colonization capacity of serotypes 19A and 6C should therefore be taken into account in the design of new pneumococcal conjugated vaccines.

A 13-valent pneumococcal conjugate vaccine (PCV13) has recently been licensed for use in children and adults at risk or with immunocompromising conditions (Advisory Committee on Immunization Practices, 2012). PCV13 is identical in formulation to PCV7 but with six additional targets covered (1, 3, 5, 6A, 7F, and 19A). As observed for PCV7, the widespread use of PCV13 will likely produce a decline in IPD caused by the serotypes covered, as well as that inflicted by certain related serotypes, e.g., serotype 6C (McEllistrem and Nahm, 2012). In fact, a recent study carried out in 8 children hospitals in the United States has shown that IPD decreased 42% overall and 53% for

children <24 months of age in 2011 compared with the 3 years before PCV13 use (Kaplan et al., 2013). Unfortunately, it remains unknown whether this vaccine can protect against infection by serotype 6D; the recent emergence of multidrug-resistant 6D pneumococci is a cause of much concern (Ko *et al*., 2012). Other non-PCV13 serotypes that appear to be rapidly increasing in prevalence include 24F (Ardanuy *et al*., 2012), 35B (Richter *et al*., 2013), 33F, 22F, 12, 15B, 15C, 23A and 11 (Kaplan et al., 2013). As shown in the present work, serotype 24F isolates do not usually form biofilms *in vitro*. The ability of serotypes 35B, 33F, 22F, 12, 15B, 15C, 23A and 11 pneumococci to produce biofilms was not investigated. The huge variety of pneumococcal serotypes that exist warrants studies on non-PCV13 emerging serotypes, which should be carefully monitored. The capacity to form biofilms, which the present work shows can be rapidly tested *in vitro,* could help in predicting the expansion of post-PCV13 pneumococcal isolates.

Experimental procedures

Strains, plasmids, media and genetic transformation

The pneumococcal strains of the different serotypes used in this study are listed in Table 1. All strains were grown in C medium (Lacks and Hotchkiss, 1960) supplemented (C+Y medium), or not, with 0.08% yeast extract. The plasmid pDLP49, which encodes the Tts β-glucosyltransferase of *S. pneumoniae* responsible for the synthesis of type 37 CPS, has been previously described (Llull *et al*., 1999; Llull *et al*., 2001). *S. pneumoniae* was transformed with chromosomal or plasmid DNA by treating precompetent cells with 100 ng/mL of synthetic competence-stimulating pheromone 1 at 37°C for 10 min to induce competence, followed by incubation at 30°C during DNA uptake. Encapsulated transformants of strain M11 were enriched by successive transfers of the transformed culture to C medium containing 0.08% bovine serum albumin, and supplemented with 0.5 μL/mL of anti-R antiserum before plating (Mollerach *et al*., 1998). Anti-R (antisomatic) antiserum contains group-specific agglutinins, which, at the

proper dilution, agglutinate only unencapsulated pneumococci. Serotyping by the capsular reaction (Quellung) was kindly performed by L. Vicioso (Spanish Pneumococcal Reference Laboratory, Majadahonda, Spain).

Biofilm formation assay and quantification

The optimal conditions for biofilm formation by pneumococcal cells have been previously described (Moscoso *et al*., 2006). In short, all biofilms were formed in Costar 3595 96-well PST microtiter plates (Corning). Cells were grown in CpH8 medium to an optical density at 550 nm (OD_{550}) of 0.5–0.6, sedimented by centrifugation, resuspended in an equal volume of CpH8 medium, and diluted 1/10. Two hundred microliter aliquots were dispensed into each well. After 6 h of incubation at 34°C, the biofilm formed was stained with 0.2% crystal violet and rinsed three times with distilled water to remove nonadherent bacteria. After solubilizing the biofilm in 95% ethanol (200 µl per well), the OD_{595} was determined using an Anthos 2020 microplate absorbance reader (Anthos Labtec Instruments).The human antibody Dob1 was diluted 1/10 to 1/40 and used to test for the inhibition of biofilm formation was kindly provided by M. H. Nahm (University of Alabama at Birmingham, AL, USA).

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed as described elsewhere (Enright and Spratt, 1998) with the tools provided by the MLST website (http://www.mlst.net).

Acknowledgements

This work was funded by the Spanish Ministerio de Economía y Competitividad (grant number SAF2012-39444-C02-01). The CIBER de Enfermedades Respiratorias (CIBERES) is an initiative of the Instituto de Salud Carlos III. The authors thank Rubén López for helpful comments and for critically reading the manuscript, Adrian Burton for revising the English version and Eloisa Cano and Susana Ruiz for skilful technical

assistance. None of the authors have any conflict of interests.

References

Advisory Committee on Immunization Practices (2012) Use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal polysaccharide vaccine for adults with immunocompromising conditions: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* **61**: 816–819.

American Academy of Pediatrics – Committee on Infectious Diseases (2000) Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis. *Pediatrics* **106**: 362–366.

Ardanuy, C., Marimón, J.M., Calatayud, L., Giménez, M., Alonso, M., Grau, I. et al. (2012) Epidemiology of invasive pneumococcal disease in older people in Spain (2007–2009): implications for future vaccination strategies. *PLoS One* **7**: e43619.

Beynon, L.M., Richards, J.C., Perry, M.B., and Kniskern, P.J. (1992) Antigenic and structural relationships within group 19 *Streptococcus pneumoniae*: chemical characterization of the specific capsular polysaccharides of types 19B and 19C. *Can J Chem* **70**: 218–232.

Bratcher, P.E., Kim, K.-H., Kang, J.H., Hong, J.Y., and Nahm, M.H. (2010) Identification of natural pneumococcal isolates expressing serotype 6D by genetic, biochemical and serological characterization. *Microbiology* **156**: 555–560.

Burton, R.L., and Nahm, M.H. (2012) Development of a fourfold multiplexed opsonophagocytosis assay for pneumococcal antibodies against additional serotypes and discovery of serological subtypes in *Streptococcus pneumoniae* serotype 20. *Clin Vaccine Immunol* **19**: 835–841.

Centers for Disease Control and Prevention (CDC) (2013) Advisory Committee on Immunization Practices (ACIP) recommended immunization schedules for persons

aged 0 through 18 years and adults aged 19 Years and Older — United States, 2013. *MMWR Surveill Summ* **62 (Suppl. 1)**: 1–19.

Chang, J., Serrano, Y., Garrido, R., Rodríguez, L.M., Pedroso, J., Cardoso, F. et al. (2012) Relevance of O-acetyl and phosphoglycerol groups for the antigenicity of *Streptococcus pneumoniae* serotype 18C capsular polysaccharide. *Vaccine* **30**: 7090–7096.

Croucher, N.J., Harris, S.R., Fraser, C., Quail, M.A., Burton, J., van der Linden, M. et al. (2011) Rapid pneumococcal evolution in response to clinical interventions. *Science* **331**: 430–434.

Dagan, R., Givon-Lavi, N., Leibovitz, E., Greenberg, D., and Porat, N. (2009) Introduction and proliferation of multidrug-resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. *J Infect Dis* **199**: 776–785.

Domenech, M., García, E., and Moscoso, M. (2009) Versatility of the capsular genes during biofilm formation by *Streptococcus pneumoniae*. *Environ Microbiol* **11**: 2542–2555.

Domenech, M., García, E., and Moscoso, M. (2012) Biofilm formation in *Streptococcus pneumoniae*. *Microb Biotechnol* **5**: 455–465.

Enright, M.C., and Spratt, B.G. (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**: 3049–3060.

Hammerschmidt, S., Wolff, S., Hocke, A., Rosseau, S., Müller, E., and Rohde, M. (2005) Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* **73**: 4653–4667.

Hanage, W.P., Bishop, C.J., Huang, S.S., Stevenson, A.E., Pelton, S.I., Lipsitch, M., and Finkelstein, J.A. (2011) Carried pneumococci in Massachusetts children: the contribution of clonal expansion and serotype switching. *Pediatr Infect Dis J* **30**: 302–308.

Hathaway, L.J., Brugger, S.D., Morand, B., Bangert, M., Rotzetter, J.U., Hauser, C. et

al. (2012) Capsule type of *Streptococcus pneumoniae* determines growth phenotype. *PLoS Pathog* **8**: e1002574.

Hausdorff, W., Hoet, B., and Schuerman, L. (2010) Do pneumococcal conjugate vaccines provide any cross-protection against serotype 19A? *BMC Pediatr* **10**: 4.

Henrichsen, J. (1995) Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* **33**: 2759–2762.

Hulten, K.G., Kaplan, S.L., Lamberth, L.B., Barson, W.J., Romero, J.R., Lin, P.L. et al. (2013) Changes in *Streptococcus pneumoniae* serotype 19A invasive infections in children from 1993 to 2011. *J Clin Microbiol* **51**: 1294–1297.

Hyams, C., Camberlein, E., Cohen, J.M., Bax, K., and Brown, J.S. (2010) The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun* **78**: 704–715.

Kaplan, S.L., Barson, W.J., Lin, P.L., Romero, J.R., Bradley, J.S., Tan, T.Q. et al. (2013) Early trends for invasive pneumococcal infections in children after the introduction of the 13-valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* **32**: 203–207.

Ko, K.S., Baek, J.Y., and Song, J.-H. (2012) Multidrug-resistant *Streptococcus pneumoniae* serotype 6D clones in South Korea. *J Clin Microbiol* **50**: 818–822.

Ko, K.S., Baek, J.Y., and Song, J.-H. (2013) Capsular gene sequences and genotypes of "serotype 6E" *Streptococcus pneumoniae* isolates. *J Clin Microbiol* **51**: 3395–3399.

Lacks, S., and Hotchkiss, R.D. (1960) A study of the genetic material determining an enzyme activity in *Pneumococcus*. *Biochim Biophys Acta* **39**: 508–518.

Laufer, A.S., Thomas, J.C., Figueira, M., Gent, J.F., Pelton, S.I., and Pettigrew, M.M. (2010) Capacity of serotype 19A and 15B/C *Streptococcus pneumoniae* isolates for experimental otitis media: Implications for the conjugate vaccine. *Vaccine* **28**: 2450–2457.

Liñares, J., Ardanuy, C., Pallares, R., and Fenoll, A. (2010) Changes in antimicrobial resistance, serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period. *Clin Microbiol Infect* **16**: 402–410.

Llull, D., García, E., and López, R. (2001) Tts, a processive *β*-glucosyltransferase of *Streptococcus pneumoniae*, directs the synthesis of the branched type 37 capsular polysaccharide in pneumococcus and other Gram-positive species. *J Biol Chem* **276**: 21053–21061.

Llull, D., Muñoz, R., López, R., and García, E. (1999) A single gene (*tts*) located outside the *cap* locus directs the formation of *Streptococcus pneumoniae* type 37 capsular polysaccharide: type 37 pneumococci are natural, genetically binary strains. *J Exp Med* **190**: 241–251.

McEllistrem, M.C., and Nahm, M.H. (2012) Novel pneumococcal serotypes 6C and 6D: anomaly or harbinger. *Clin Infect Dis* **55**: 1379–1386.

Mollerach, M., López, R., and García, E. (1998) Characterization of the *galU* gene of *Streptococcus pneumoniae* encoding a uridine diphosphoglucose pyrophosphorylase: a gene essential for capsular polysaccharide biosynthesis. *J Exp Med* **188**: 2047–2056.

Moscoso, M., García, E., and López, R. (2006) Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol* **188**: 7785–7795.

Nuorti, J.P., Whitney, C.G., and Centers for Disease Control and Prevention (CDC) (2010) Prevention of pneumococcal disease among infants and children — Use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal polysaccharide vaccine. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* **59**: 1–18.

O'Brien, K.L., Wolfson, L.J., Watt, J.P., Henkle, E., Deloria-Knoll, M., McCall, N. et al. (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**: 893–902.

Oliver, M.B., van der Linden, M.P.G., Küntzel, S.A., Saad, J.S., and Nahm, M.H. (2013a) Discovery of *Streptococcus pneumoniae* serotype 6 variants with glycosyltransferases synthesizing two differing repeating units. *J Biol Chem* **288**: 25976–25985.

Oliver, M.B., Jones, C., Larson, T.R., Calix, J.J., Zartler, E.R., Yother, J., and Nahm, M.H. (2013b) *Streptococcus pneumoniae* serotype 11D has a bispecific glycosyltransferase and expresses two different capsular polysaccharide repeating units. *J Biol Chem* **288**: 21945–21954.

Park, S., Parameswar, A.R., Demchenko, A.V., and Nahm, M.H. (2009) Identification of a simple chemical structure associated with protective human antibodies against multiple pneumococcal serogroups. *Infect Immun* **77**: 3374–3379.

Richter, S.S., Heilmann, K.P., Dohrn, C.L., Riahi, F., Diekema, D.J., and Doern, G.V. (2013) Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999–2011. *Emerg Infect Dis* **19**: 1074–1083.

Rodgers, G.L., Arguedas, A., Cohen, R., and Dagan, R. (2009) Global serotype distribution among *Streptococcus pneumoniae* isolates causing otitis media in children: potential implications for pneumococcal conjugate vaccines. *Vaccine* **27**: 3802–3810.

Spijkerman, J., van Gils, E.J.M., Veenhoven, R.H., Hak, E., Yzerman, E.P.F., van der Ende, A. et al. (2011) Carriage of *Streptococcus pneumoniae* 3 years after start of vaccination program, the Netherlands. *Emerg Infect Dis* **17**: 584–591.

Sun, Y., Park, M.K., Kim, J., Diamond, B., Solomon, A., and Nahm, M.H. (1999) Repertoire of human antibodies against the polysaccharide capsule of *Streptococcus pneumoniae* serotype 6B. *Infect Immun* **67**: 1172–1179.

Weinberger, D.M., Malley, R., and Lipsitch, M. (2011) Serotype replacement in disease after pneumococcal vaccination. *Lancet* **378**: 1962–1973.

Weinberger, D.M., Trzciński, K., Lu, Y.-J., Bogaert, D., Brandes, A., Galagan, J. et al. (2009) Pneumococcal capsular polysaccharide structure predicts serotype prevalence. *PLoS Pathog* **5**: e1000476.

Whitney, C.G., Farley, M.M., Hadler, J., Harrison, L.H., Bennett, N.M., Lynfield, R. et al. (2003) Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**: 1737–1746.

Wolcott, R.D., and Ehrlich, G.D. (2008) Biofilms and chronic infections. *JAMA* **299**:

2682–2684.

Table 1. *S. pneumoniae* strains used.

Strain	Description[a]	Source or reference[b]
M11	R6 derivative (nonencapsulated)	(Moscoso et al., 2006)
CLINICAL		
1037/10	19F; blood; ST9077	A. Fenoll; ISCIII
1064/10	19F; AOM; ST177	A. Fenoll; ISCIII
2567/10	19F; CSF; ST9078	A. Fenoll; ISCIII
2789/10	19F; BAA; ST9079	A. Fenoll; ISCIII
SSISP19F/1	19F	Statens Serum Institut
G54	19F; ST63	(Moscoso et al., 2006)
1041/10	19A; blood; ST9065	A. Fenoll; ISCIII
1061/10	19A; AOM; ST9066	A. Fenoll; ISCIII
2797/10	19A; BAA; ST9080	A. Fenoll; ISCIII
2998/10	19A; CSF; ST2013	A. Fenoll; ISCIII
5086	19A; ST81	J. Liñares; IDIBELL
82220	19B; ST3492	Statens Serum Institut
82211	19C; ST9081	Statens Serum Institut
1356/12	6A; ST2467	A. Fenoll; ISCIII
1056/12	6B; ST179	A. Fenoll; ISCIII
1262/12	6C; ST386	A. Fenoll; ISCIII
840/12	18A; ST9082	A. Fenoll; ISCIII
910798	18B; ST113	H. Bootsma, Radboud Univ.
854/12	18C; ST9083	A. Fenoll; ISCIII
SSISP18C/1	18C; ST4706	Statens Serum Institut
2020/11	24F; ST230	A. Fenoll; ISCIII
TRANSFORMANTS		
P014	M11 transformed with SSISP18C/1 DNA (18C)	This study
P015	M11 derivative (23F[c])	(Moscoso et al., 2006)
P181	M11 transformed with 5086 DNA (19A)	This study
P182	M11 transformed with 82220 DNA (19B)	This study
P184	M11 transformed with 82221 DNA (19C)	This study
P191	M11 transformed with 1064 DNA (19F)	This study
P192	M11 transformed with G54 DNA (19F)	This study

P194	P192 transformed with pDLP49 (19F/37; binary)	This study
P198	M11 transformed with 1356/12 DNA (6A)	This study
P199	M11 transformed with 1056/12 DNA (6B)	This study
P200	M11 transformed with 1262/12 DNA (6C)	This study
P201	M11 transformed with 840/12 DNA (18A)	This study
P202	M11 transformed with 854/12 DNA (18C)	This study
P224	M11 transformed with 2020 DNA (24F)	This study
P230	M11 transformed with 910798 DNA (18B)	This study

a. AOM, acute otitis media, BAA, bronchoalveolar aspirate; CSF, cerebrospinal fluid.

b. IDIBELL, Instituto de Investigación Biomédica de Bellvitge, Barcelona, Spain; ISCIII, Instituto de Salud Carlos III, Majadahonda (Madrid), Spain.

c. This strain was originally reported as 19F. However, we have found that it actually corresponds to a 23F transformant (unpublished).

Figure Legends

Fig. 1. Primary structures and antigenic formulae for the serotypes belonging to *S. pneumoniae* serogroup 19.

The known structures of serogroup 7 CPSs are also shown for comparison. The initial sugar attached to the undecaprenyl phosphate lipid carrier is shown on the right. The trisaccharide common to all serogroup 19 polysaccharides is boxed. The predicted 7a antigen common to serogroup 7 pneumococci is shown in the dotted rectangle. The proposed disaccharide corresponding to the 7h antigen common to type serotypes 19B, 19C and 7B is shown in the grey box.

Fig. 2. Biofilm formation by encapsulated *S. pneumoniae* strains of serogroup 19.

Clinical isolates of this serogroup were grown in C medium for 6 h at 34°C. Biofilm formation (solid bars) was quantified by staining with crystal violet. Grey bars correspond to biofilms developed by encapsulated transformants of the indicated serotype using the unencapsulated M11 strain as a recipient. The M11 strain was used as a control (open bar). Biofilm formation by encapsulated strains was normalized for absorbance, and the percentage normalized against the control strain M11. The percentages shown are the mean ± standard error of at least four independent experiments performed in triplicate.

Fig. 3. Capsular polysaccharide and biofilm formation by encapsulated *S. pneumoniae* strains of serogroup 6.

A. Primary structures of the CPS of the different serotypes of serogroup 6. The disaccharide shared with serotype 19A is marked in black.

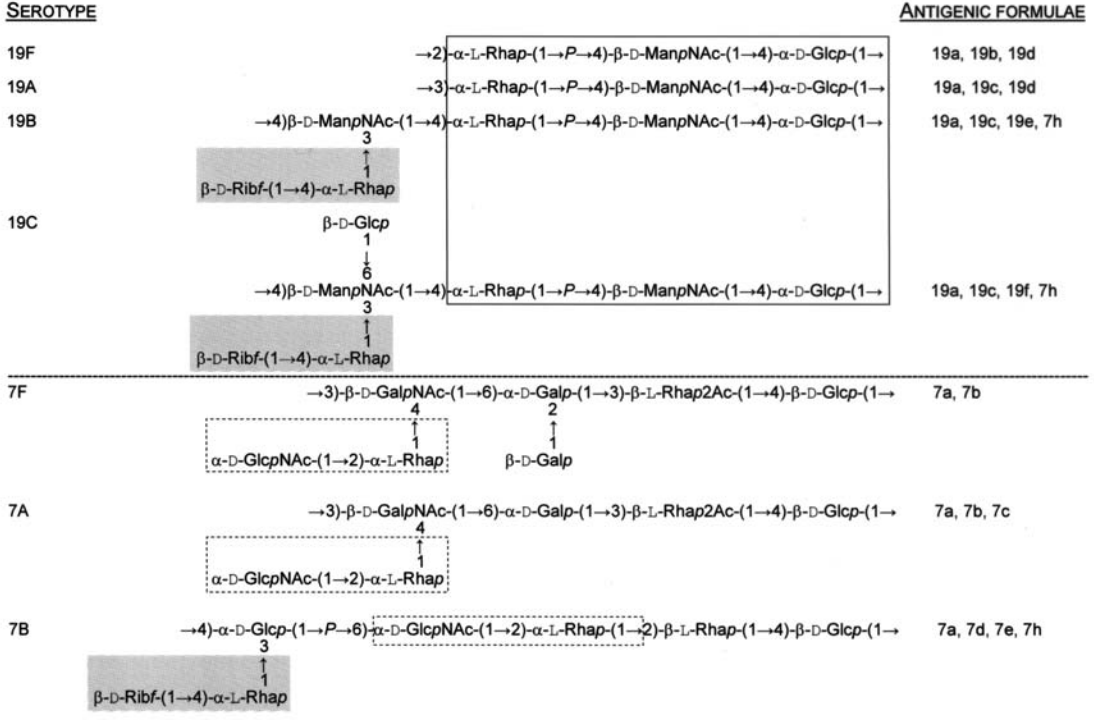
B. Biofilm formation assay. Growth conditions and biofilm quantification techniques were the same as for serogroup 19. Open bar - control strain M11; solid bars - clinical isolates; grey bars - encapsulated transformants of the indicated serotype using the unencapsulated M11 strain as a recipient. Biofilm formation by the encapsulated strains

was normalized for absorbance, and the percentage normalized against the control strain M11. The percentages shown are the mean ± standard error of at least three independent experiments performed in triplicate.

Fig. 4. Capsular polysaccharides and biofilm formation by encapsulated *S. pneumoniae* strains of serogroup 18.

A. Primary structures of capsular polysaccharides of the different serotypes of serogroup 18. A disaccharide very similar to the one present in serotype 19A is marked in black.

B. Biofilm formation assay. Growth conditions and biofilm quantification techniques were the same as for serogroup 19. Open bar - control strain M11; solid bars - clinical isolates; grey bars - encapsulated transformants of the indicated serotype using the unencapsulated M11 strain as a recipient. Biofilm formation by the encapsulated strains was normalized for absorbance, and the percentage normalized against the control strain M11. The percentages shown are the mean ± standard error of at least three independent experiments performed in triplicate.

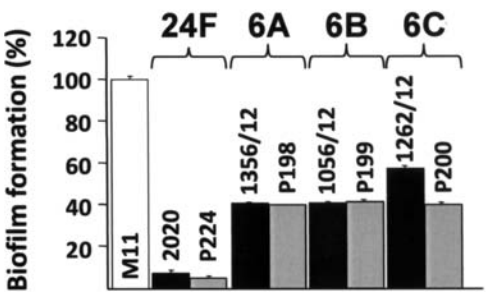

SEROTYPE
ANTIGENIC FORMULAE
19F
→2)-α-L-Rhap-(1→P→4)-β-D-ManpNAc-(1→4)-α-D-Glcp-(1→
19a, 19b, 19d
19A
→3)-α-L-Rhap-(1→P→4)-β-D-ManpNAc-(1→4)-α-D-Glcp-(1→
19a, 19c, 19d
19B
→4)β-D-ManpNAc-(1→4)-α-L-Rhap-(1→P→4)-β-D-ManpNAc-(1→4)-α-D-Glcp-(1→
19a, 19c, 19e, 7h
3
1
β-D-Ribf-(1→4)-α-L-Rhap
19C
β-D-Glcp
1
6
→4)β-D-ManpNAc-(1→4)-α-L-Rhap-(1→P→4)-β-D-ManpNAc-(1→4)-α-D-Glcp-(1→
19a, 19c, 19f, 7h
3
1
β-D-Ribf-(1→4)-α-L-Rhap
7F
→3)-β-D-GalpNAc-(1→6)-α-D-Galp-(1→3)-β-L-Rhap2Ac-(1→4)-β-D-Glcp-(1→
7a, 7b
4
2
1
1
α-D-GlcpNAc-(1→2)-α-L-Rhap
β-D-Galp
7A
→3)-β-D-GalpNAc-(1→6)-α-D-Galp-(1→3)-β-L-Rhap2Ac-(1→4)-β-D-Glcp-(1→
7a, 7b, 7c
4
1
α-D-GlcpNAc-(1→2)-α-L-Rhap
7B
→4)-α-D-Glcp-(1→P→6)-α-D-GlcpNAc-(1→2)-α-L-Rhap-(1→2)-β-L-Rhap-(1→4)-β-D-Glcp-(1→
7a, 7d, 7e, 7h
3
1
β-D-Ribf-(1→4)-α-L-Rhap

Biofilm formation (%)
120
100
80
60
40
20
23F
19F
19F + 37
19A
19B
19C
M11
P015
1037
1064
P191
2567
2789
SSISP19F/1
G54
P192
P194
1041
1061
2797
2998
5086
P181
82220/19B
P182
82221/19C
P184

A

Serotype		Antigenic formulae
19F	→**2)**-α-L-**Rha*p*-(1**→*P*→4)-β-D-Man*p*NAc-(1→4)-α-D-**Glc*p*-(1**→	19a, 19b, 19d
19A	→**3)**-α-L-**Rha*p*-(1**→*P*→4)-β-D-Man*p*NAc-(1→4)-α-D-**Glc*p*-(1**→	19a, 19c, 19d
6A	→**3)**-α-L-**Rha*p*-(1**→3)-D-Rib-ol-(5→*P*→2)-α-D-Gal*p*-(1→3)-α-D-**Glc*p*-(1**→	6a, 6b
6B	→**3)**-α-L-**Rha*p*-(1**→4)-D-Rib-ol-(5→*P*→2)-α-D-Gal*p*-(1→3)-α-D-**Glc*p*-(1**→	6a, 6c
6C	→**3)**-α-L-**Rha*p*-(1**→3)-D-Rib-ol-(5→*P*→2)-α-D-Glc*p*-(1→3)-α-D-**Glc*p*-(1**→	6a, 6b, 6d
6D	→**3)**-α-L-**Rha*p*-(1**→4)-D-Rib-ol-(5→*P*→2)-α-D-Glc*p*-(1→3)-α-D-**Glc*p*-(1**→	6a, 6c, 6d

B



A

Serotype		Antigenic formulae
19A	→3)-α-L-Rha*p*-(1→*P*→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→	19a, 19c, 19d
18A	D-Gro-(1→*P* ↓ 3 →3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→ 2 ↑ 1 α-D-Glc*p*	18a, 18b, 18d
18B	D-Gro-(1→*P* ↓ 3 →3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→ 2 ↑ 1 α-D-Glc*p*	18a, 18b, 18e, 18g
18C	D-Gro-(1→*P* ↓ 3 →3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*(1→ 2 ↑ 1 α-D-Glc*p*6Ac$_{0.3}$	18a, 18b, 18c, 18e

B